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ELI LILLY AND COMPANY

By_____ Date_____

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Mark Brader, *et al.*

Serial No.: 08/484,542

Filed: June 7, 1995

For: **Stabilized, Acylated Insulin Formulations**

Docket No.: X-10097

Group Art Unit: 1631

Examiner: M. Allen

DECLARATION OF DR. AHMED DELDAR UNDER 37 C.F.R. § 1.608(b)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Ahmed Deldar declares as follows:

1. I am an employee of Eli Lilly and Company, the assignee of this patent application. I have been employed at Eli Lilly and Company since 1986. I received a Doctor of Veterinary Medicine degree from Pahlavi University in 1978. I received a doctorate in Medical Sciences from the University of Pennsylvania in 1986.

2. I am not a co-inventor in this application. I provide this declaration in order to provide confirmation that in dose ranging study number D06893, compound LY309132 with zinc exerted a hypoglycemic effect *in vivo*.

3. Exhibit 16 is a photocopy of a clinical pathology report for study number D06893. The title of the protocol was "A Dose Ranging Study with LY309132 Administered Subcutaneously to Beagle Dogs." I generated this document after study D06893 was completed, in order to explain the effect of LY309132 on blood glucose and blood triglyceride levels in beagle dogs.

4. In this *in vivo* study, blood was collected for glucose and triglyceride determinations from each animal at 0, 0.5, 1, 2, 4, 6 and 24 hours after LY309132 dose administration on Day 0, at 0, 0.5, 1, 2, 4, 6, 8 and 24 hours after LY309132 dose administration on Day 5, and at 0, 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after LY309132 dose administration on Days 12 and 19.

5. At page 1 of the report, I discuss the administration of 0.07, 0.11 and 0.2 mg/kg of LY309132, and I explain the hypoglycemic effect of each dose.

6. For the 0.07 mg/kg dose, I explain that it resulted in a slight decrease in mean blood glucose (*ca* 9%) at 30 minutes post-injection. Thereafter, mean blood glucose fluctuated and continued to decrease, reaching its maximum drop (*ca* 31 and 43% in males and females, respectively) by 4 hours post-injection, compared to the pretreatment values. Mean blood glucose began to rise thereafter, and reached above the pretreatment values (*ca* 26%) at 24 hours post-injection.

7. For the 0.11 mg/kg dose, I explain that it resulted in less remarkable changes in mean blood glucose levels, compared to the earlier dose of 0.07 mg/kg. Maximum decrease in mean blood glucose (*ca* 17%) occurred at 8 hours post-injection. By 24 hours post-injection, mean blood glucose level was above the pretreatment values (*ca* 27%).

8. For the 0.2 mg/kg dose, I explain that it resulted in a progressive decrease in mean blood glucose levels. An initial minimal decrease (*ca* 4%) at 30 minutes post-injection reached its maximum drop in males (*ca* 44%) and females (*ca* 33%) by 8 hours and 4 hours post-injection, respectively, compared to the pretreatment values. Thereafter, mean blood glucose level began to rise, but remained below the pretreatment values (*ca* 4%) by 24 hours post-injection in both males and females.

9. At page 2 of the report, I explain that a 0.2 mg/kg dose of LY309132 containing zinc revealed relatively similar changes as I describe for the 0.2 mg/kg dose without zinc. I also explain that maximum effects on blood glucose level occurred following the administration of 0.2 mg/kg LY309132 with or without addition of zinc.

10. At page 2 of the report, I explain that administration of LY309132 resulted in an overall dose-related increase in mean serum triglycerides. The increases were largely noted at 30 minutes post-injection and were either progressive, fluctuated or sustained through the next 6 hours post-injection. The increases in mean serum triglycerides became comparatively less remarkable thereafter, and the mean values were either comparable to or less than the pretreatemnt values by 24 hours post-injection. The increases in mean serum triglycerides following the administration of 0.11 mg/kg LY309132 were exaggerated, because of the pretreatment values which were lower than the established reference ranges for the species.

10. I signed the report on page two on April 7, 1994. I confirm that the handwriting in the signature is mine. My title at that time was Senior Clinical Pathologist.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information of belief are believed to be true; and I am warned that all statements made herein were made with the knowledge that willful false statements are punishment by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful and false statements may jeopardize the validity of any patent issued from this application.

Ahmed Deldar

April 23, 2003

From Page No. ___

8/9/93

Samples for Lyophilization Studies.

Aim: To investigate the reconstitutability of B29(C16) from three different lots after being lyophilized. Also to investigate the effect of Zn.

Samples from lots # DBF40, BXG203 and BXG209 were investigated.

Method: Want to lyophilize 10 5ml vials (# BT5846) containing 1ml of solution at 5.0 mg/ml, pH: 7.4. Dissolve solid in 0.01N HCl and titrate thru isoelectric point with 0.2N NaOH.

BXG203.

72.0mg were dissolved in 10mls 0.01N HCl. 730µl of 0.2N NaOH was required to acheive a final pH of 7.39 Added 2.27mls H_2O to give a final volume of 13mls.

Assuming a 90% potency, expect 4.98 mg/ml C16

From UV. A_{280}: 37032 (80µl/ml) = 5.3 mg/ml protein

Final pH = 7.39

Sol^n not filtered prior to giving to M. Roy for filtering, filling and freeze-drying.

To Page No.

Rec rded by:	Date	Verified by:	Date
M. L. Brader	10/12/93		

Project No. H4R
Book N. _____ TITLE LY309132

From Page No. ___

DBF40 Dissolved 112.4 mg in 18 mls 0.01N HCl. This solution was divided into two 9.050 ml samples.

To the 9.050 mls sample 870 µl of 0.2M NaOH was added to produce a pH of 7.42. To this solution a further 350 µl of water was added to give a final volume of 10.25 mls.

Assuming a 90% potency expect : 4.93 mg/ml C16

By UV. A_{280} = 0.18282 (40µl+1ml) = 5.04 mg/ml protein

DBF40Zn To the 9.050 mls 60.9 µl of a 3.91 mg/ml ZnO solution was added.

Zn^{2+} sol^{n} 39.1 mg of ZnO was dissolved in 5.0 mls 1N HCl then made up to 10 mls with H_2O.

Zn : C16 mole ratio = 0.35 assuming 90% potency of the C16 solid.

The solution remained clear after the addition of Zn^{2+}. 1040 µl of 0.2M NaOH was added to reach a pH of 7.42.

Added 100 µl to give a final vol. of 10.25 mls.

Assuming 90% potency expect 4.93 mg/ml C16

From UV. A_{280} = .3703 (80µl+1ml) : 5.3 mg/ml

To Pag No. ___

Recorded by:	Date	Verified by:	Date
M L Brooks	9/9/93	M Beckage 10/12/93	

From Page No.___

BXG209 Most recently available lot, provided by B. Moser.
85% protein by wt.] from HPLC.
80% (C16)B29 by wt.]

Dissolved 65.4 mg in 9 mls 0.01 N HCl.
Added 690 µl of 0.2 M NaOH to give a pH. 7.41
Added 1.310 mls to give a final Vol. of 11.0 mls

Expect for 85% potency 5.05 mg/ml
" for 80% " 4.76 mg/ml

From UV. A_{280}: .38425 5.50 mg/ml

Final pH= 7.41, not filtered.

These samples were provided to Michael Roy, 2:00 pm 9/9/93 for filtering (0.45µ filter), filling into 5 ml vials # BTS846 and freeze drying according to the procedure followed by Steve Bradner for the reference standard.

Summary

C16lyophilization

	Lot #	Weight, mg	Volume, ml	Potency, %	C16 conc, mg/ml	Protein Conc.(UV)	pH
1	BGX203	72	13	90	4.98	5.3	7.39
2	DBF40	56.2	10.25	90	4.93	5.04	7.42
3	DBF40Zn	56.2	10.25	90	4.93	5.3	7.42
4	BXG209	65.4	11	80	4.76	5.5	7.41

~~9/9/93~~ 9/10/93

Reconstitution: The reconstitution experiment was conducted by adding 1 ml of Humulin R diluent to a vial containing the lyophilized material. In each sample the lyophilized C16 material dissolved completely in less than 30 seconds yielding a clear solution.

To Page No.___

Recorded by:	Date	Verified by	Date
MR Bradner	9/10/93	M Beckage 10/12/93	

From Page No.___

9/28/93 Preparation of Solutions of CT Material for Lyophilization

Method: 1.20g of C16-insulin (lot #487EM3) was dissolved in 15.8mls of 0.01N HCl. Required 30 minutes to dissolve. Divided into two 7.5ml aliquots.

ZnO sol^n Dissolved 0.240g ZnO (QA053A) in 10mls 1N HCl then made up to 100mls.
= 2.4 mg/ml

Zn : C16 ratio. 600mg of C16-insulin
85% potency
= 510mg = 8.44×10^{-5} moles
$\Rightarrow$ require $0.35 \times 8.44 \times 10^{-5}$ moles of ZnO
$= 2.952 \times 10^{-5}$ moles ZnO

∴ Require = 2.40 mg ZnO for 600mg of bulk

ZnO sol^n Add 1ml then pH to 7.60
Volume was adjusted to 100mls with distilled H_2O.

Result:

100mls Zn-free C16, pH: 7.60 A_{280} = .4550 (10µl in 1ml)
100mls Zn-containing C16, pH: 7.60 A_{280} = .4723 (10µl in 1ml)

(1) Zn-free : 5.31 mg/ml by uv 2685-47B
Zn-containing : 5.51 mg/ml by uv 2685-47A

To Page No.___

Rec rded by:	Date	Verified by:	Date
M. Beckage	9/28/93	M. Beckage	10/12/93

From Page No.

These unfiltered solutions were provided to Michael Ray for lyophilization at 4:00pm 9/28/93.
Expt# 2 x 100 vials each. with 1ml.

2685-47A = Zn-containing Sol^n
2685-47B = Zn-free

Amino Acid Analysis Result (Dean Codiglia)

2685-47A = 5.09 mg/vial ± 2.4% TRS
2685-47B = 5.15 mg/vial ± 2.5% TRS

3.5mg/ml = U100 (BHI)

In 1ml

$\frac{3.5 \times 10^{-3}}{5808} = 6.026 \times 10^{-7}$ moles Lys Pro in 1ml

A 0.50 mole ratio of Zn^{2+}

$\Rightarrow 0.5 \times 6.026 \times 10^{-7}$

$= 3.013 \times 10^{-7}$ moles Zn^{2+} required

$\Rightarrow 3.013 \times 10^{-7} \times 65.39$ grams Zn^{2+}

$= 1.970 \times 10^{-5}$ g Zn^{2+}

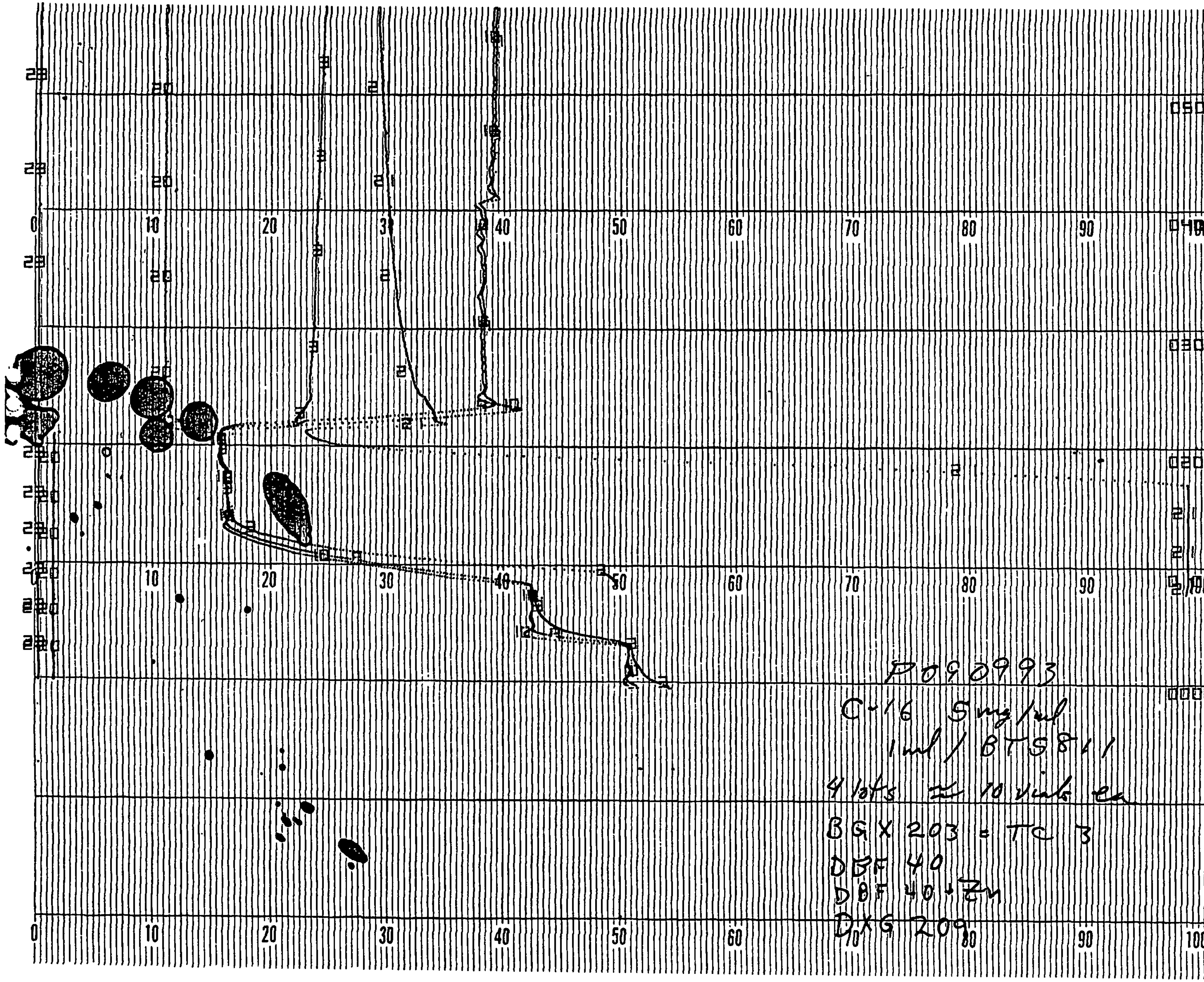
$= 19.7$ µg Zn^{2+}/ml

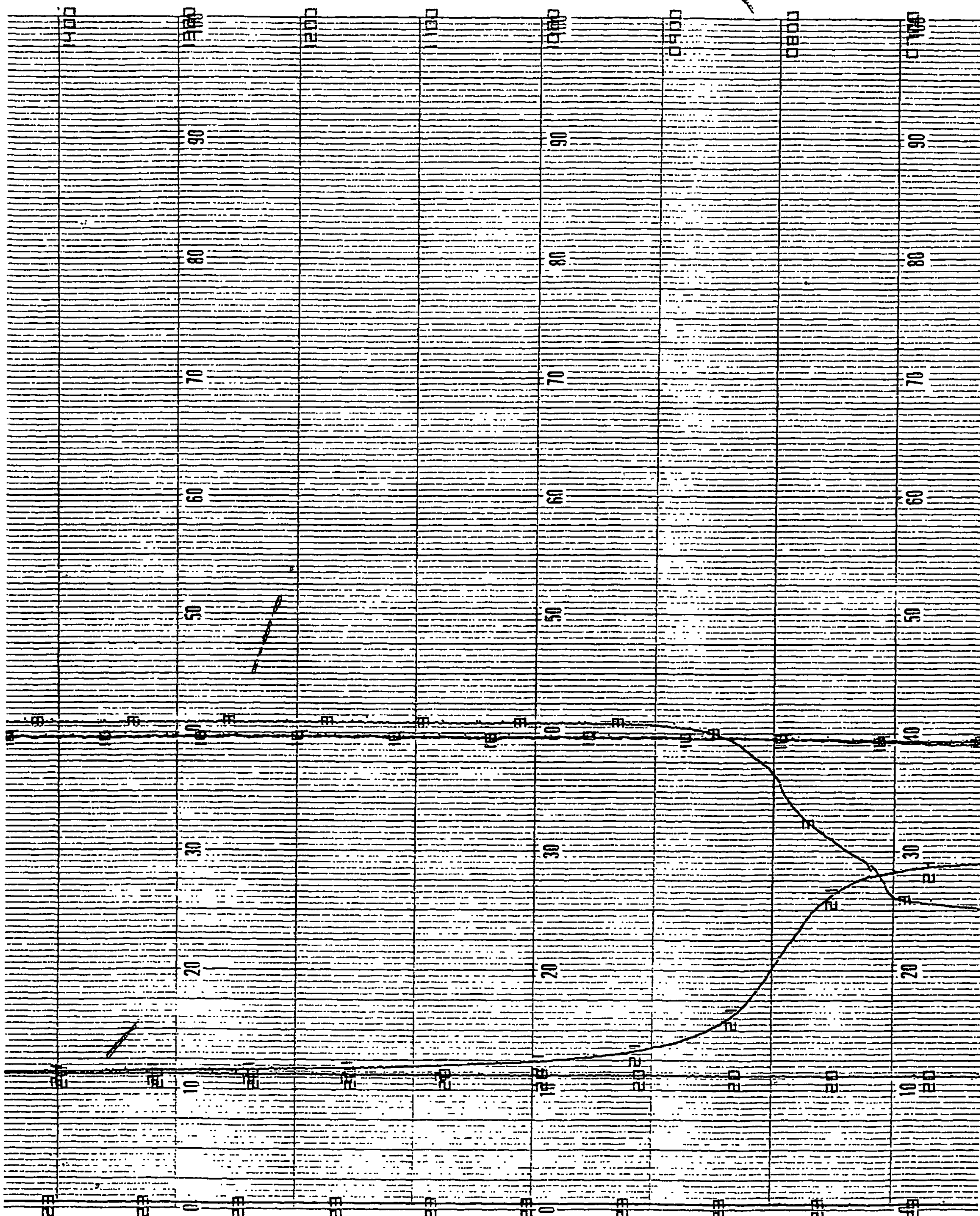
or $3.013 \times 10^{-7} \times (65.39+16)$ grams ZnO

$= 24.52$ µg ZnO /ml

To Page No.

Recorded by:	Date	Verified by:	Dat
MBrooks	9/28/93	MA Beckage 10/1/93	


P090993
C-16 5mg/ml
1ml / BTS811
4 lots ≃ 10 vials ea
BGX 203 = TC 3
DBF 40
DBF 40 + Zn
DXG 209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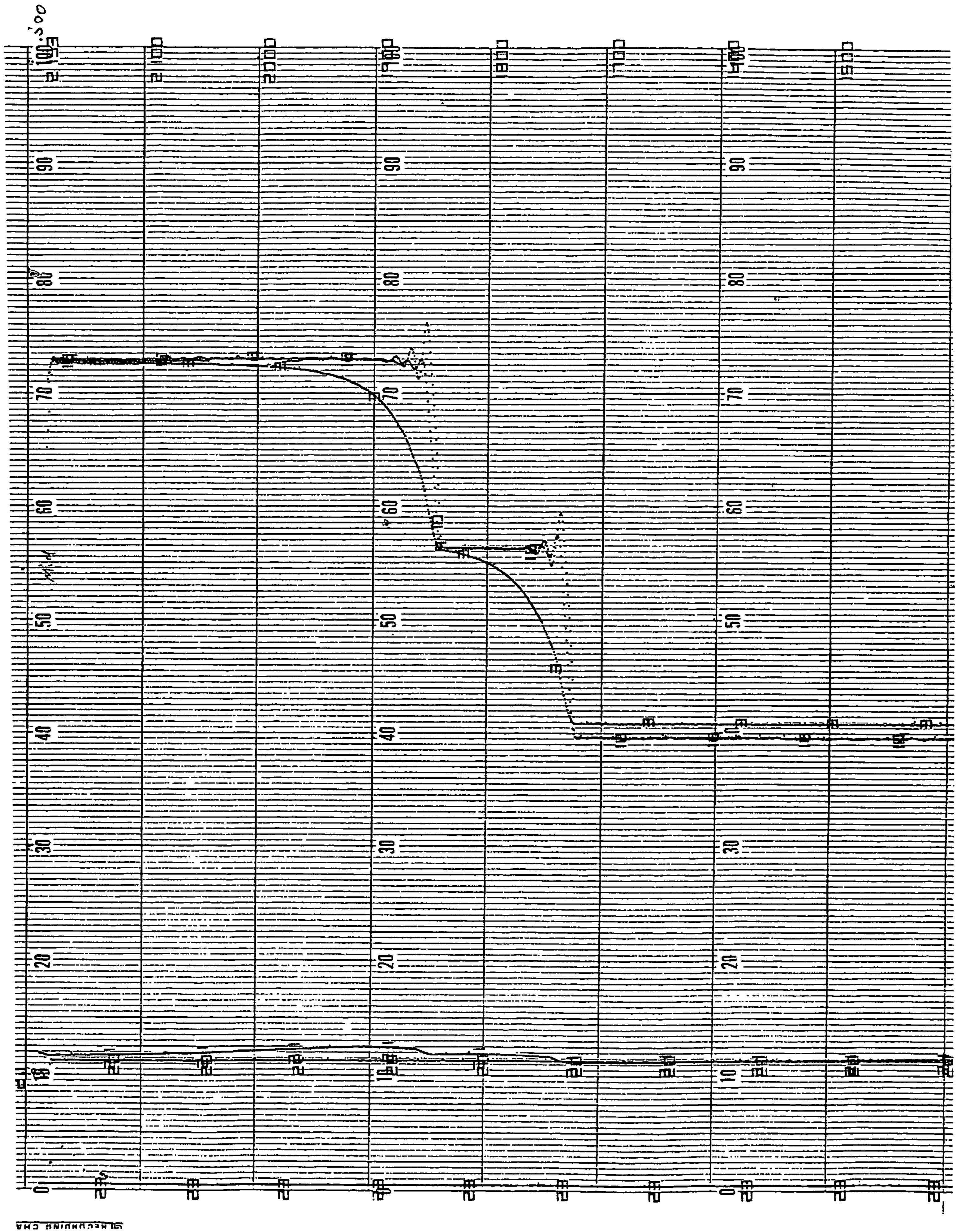

Testing Facility: Toxicology Research Laboratories
Lilly Research Laboratories
Greenfield, Indiana 46140

Sponsor: Eli Lilly and Company

A DOSE RANGING STUDY WITH LY309132 ADMINISTERED SUBCUTANEOUSLY TO BEAGLE DOGS.

1. STUDY LOG

Test Article: 309132
Print Description: LY309132

Study No.: D06893

Est. Exp. Start: 09/22/93
Est. Exp. Term.: 10/22/93

Duration: 30 (days)

Study Type: Dose Ranging

Species: Dog
Strain: Beagle

Route: Subcutaneous

Building: 240
Room: 609

Project Number: H4R

Study Design Date: 09/01/93
Est. Report Date: N/A

GLP: N

Study Director:
R.A. Byrd

Scientist Supervisor:
N.R. Bernhard

Pathologist:
None Assigned

Project Leader:
R.A. Byrd

Lead Technician:
M.J. Zeilinga

Technician:
L.R. Ebbert

Head,Study Area:
M.A. Dorato

Approved by: R.A. Byrd 9/16/93 Approved on:

Study Director R.A. Byrd 09/16/93

Scientist Supervisor N.R. Bernhard 09/16/93

2. DISTRIBUTION LIST

Archives
Mr. N. R. Bernhard (Scientist Supervisor)
Mr. R. A. Byrd (Study Director/Project Leader)
Mr. L. R. Ebbert (Technician)
Dr. N. A. Farid (Pharmacokineticist, MC909, 0825)
Dr. D. M. Hoover (Head, Morphologic Pathology)
Mr. J. A. Klepfer (Clinical Pathology)
Dr. J. P. McGrath (Clinical Pathologist)
Ms. A. B. Nauden (Report Resource Center)
Quality Assurance
Dr. G. D. Smith (Clinical Veterinarian)
Mr. S. M. Snyder (Dept. Head, Large Animal)
Mr. R. L. St Clair (Dept. Head, Toxicology Formulations)
Dr. R. R. Swain (Clinical Chemistry)
Toxicology Formulations
Mr. M. J. Zeilinga (Lead Technician)

3. RECORD PREPARATION

Histology __ Blood Levels _X_ Master Schedule _X_

4. PURPOSE OF STUDY

This study is to determine the maximum tolerated hypoglycemic dose and to measure serum concentrations of LY309132 .

5. TEST ARTICLE

Compound No.: LY309132

Analytical Characterization of Technical Material
Lot No.: RS0163
Potency: Approximately 4.91 mg LY309132 mg/vial
Date Available: Now
Est. Quant. Needed: 40 mg

Diluent: Humulin R diluent
Lot No.: 6PG22A

Periodic Reassay: No

Storage Conditions: Formulated vials of LY309132 store at -20 degrees C

Diluent store at room temperature.

Chemical Archive Sample: No

Precautions: Personnel in the Large Animal Toxicology Area will follow the guidelines in TOX-LAT-***-004 when dosing and restraining dogs. Personnel in the Toxicology Formulations Area should follow the appropriate guidelines for their area during solution preparation.

6. DOSE PREPARATION

Correct doses for potency.

Treatment Group	Dose (mg LY309132/kg)	Preparation
01	0.07	0.7/mg LY309132/ml diluent

Diluent: Humulin R diluent

pH Determination: Yes; at each concentration

Special Instructions:

1. All dose solutions will be sterile filtered with a 0.22 micron filter.

2. Additional doses may be added depending upon results of the previous dose. Additional doses will also be administered approximately 3 days apart.

7. DOSE ADMINISTRATION

Route: Subcutaneous

Frequency: Once at each dose level for all dogs.

Dose Volume per Unit Body Weight: 0.1 ml/kg

Special Instructions: Subsequent doses may be elevated or lowered from those proposed depending on blood glucose levels.

Doses will be administered into the dorsal neck area.

8. ANIMALS

A. General

1.

Treatment Group	Dose (mg/kg)	Number of Animals Males	Females
01	0.07	3	3

2. Justification of Use: The beagle dog has been used extensively in toxicity studies in this laboratory and a large amount of biological data is available.

3. Approximate Age: Adult

4. Initial Body Weight Range: 7.5 to 10.5 kg

5. Supplier: Marshall Farms, North Rose, NY

B. Individual Animal Information (attached)

9. ANIMAL SELECTION/DISTRIBUTION AND IDENTIFICATION

A. Animals will be selected/distributed to dose groups as described in Procedure Manuals TOX-LAT-***-002 and TOX-LAT-***-003.

B. Animals will be identified as described in Procedure Manual TOX-LAT-***-002.

10. ANIMAL CARE

Use Procedure Manuals TOX-DIV-HUS-001 and TOX-DIV-HUS-010.

11. FOOD

Supplier/Type: Purina/Certified Canine Diet 5007

Special Instructions:

1. Approximately 150 g of 5007 will be offered daily to each dog at about 0.5 hour prior to dose administration.

2. Dogs will be dosed only after having eaten a portion of the ration of 5007.

3. To minimize the chance of hypoglycemic shock, approximately 150 g Certified Canine Diet 5007 will be offered additionally to each dog immediately after dose administration.

4. Dogs will be fasted at least 6 hours prior to each dose administration.

5. A supplemental diet such as Hill's Prescription Diet will be offered to dogs that will not eat the Certified Canine Diet 5007.

12. OBSERVATIONS

Daily observations: Record observations as described in Procedure Manual TOX-LAT-***-002 and TOX-DIV-ADM-004.

13. BODY WEIGHT

Weigh dogs prior to each dose according to Procedure Manual TOX-LAT-***-007.

14. FOOD CONSUMPTION

Visual estimation daily according to Procedure Manual TOX-LAT-***-008.

15. CLINICAL CHEMISTRY

Determine blood glucose and triglycerides levels on all dogs at 0, 0.5, 1, 2, 4, 6 and 24 hours after each dose administration.

Glucose
Triglycerides

16. BIOCHEMICAL TOXICOLOGY

Determine serum concentration of LY309132 at 0, 0.5, 1, 2, 4, 6, and 24 hours after each dose administration.

Anticoagulant: None

Sample size: 3 ml whole blood

Send samples to: N. A. Farid (MC909, 0825)

Special Instructions:

Samples will be collected, and delivered to sample processing for separation and removal of the serum. Store serum at -70 degrees C. Ship samples frozen on dry ice.

17. RECORDS

All raw data, supporting documents, protocol, and the final report will be retained by Lilly Research Laboratories.

18. STATISTICS

Quantitative results for blood glucose and triglycerides will be analyzed for each sex at each time point with a one-factor analysis of variance. Linear treatment contrasts will be tested in a sequential fashion (Tukey, et al. 1985) to evaluate the dose response. Blood level concentrations and unexpected findings may generate alternative and/or additional statistical analyses.

=== ESTIMATED STUDY CALENDAR ===

Study Day	Date	Animals	Activity
0	WEDNESDAY SEP. 22, 1993	6	Experiment Start
		6	Clinical Chemistry
		6	Blood Level w/no sacrifice
1	THURSDAY SEP. 23, 1993	6	Clinical Chemistry
		6	Blood Level w/no sacrifice
5	MONDAY SEP. 27, 1993	6	Clinical Chemistry
		6	Blood Level w/no sacrifice
6	TUESDAY SEP. 28, 1993	6	Clinical Chemistry
		6	Blood Level w/no sacrifice
8	THURSDAY SEP. 30, 1993	6	Clinical Chemistry
		6	Blood Level w/no sacrifice
9	FRIDAY OCT. 1, 1993	6	Clinical Chemistry
		6	Blood Level w/no sacrifice

ANIMAL INFORMATION

CAGE NO.	ANIMAL NUMBER	SEX	WHELP DATE	TATTOO	SUP.	TRMNT. GROUP	TYPE DD	TERM. DATE
0233	255491	M	9/ 1/89	1490192	MR	1		
0234	256051	M	9/12/89	1497456	MR	1		
0235	273071	M	12/20/91	1931245	MR	1		
0236	255691	F	5/ 4/92	2000601	MR	1		
0237	256961	F	6/24/92	2025302	MR	1		
0238	257591	F	6/13/92	2019094	MR	1		

TREATMENT INFORMATION

GROUP		DOSE	TEST-ARTICLE	START	TERMINATION	FREQUENCY
1	A	0.07 (MG/KG)	309132	9/15/93 08:00	10/15/93	Single Dose

ROUTE: Subcutaneous

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 1 (0-24 hour bleed)

Listed below are the glucose values (mg/dl) for samples collected over a 24 hour period.

Animal #	0hr	0.5hr	1.0hr	2.0hr	4.0hr	6.0hr	24hr
256051 M	70	80	56	67	36	78	100
256961 F	79	69	80	83	53	63	88
273071 M	82	74	70	77	61	65	99
255491 M	84	65	66	70	65	80	103
255691 F	77	67	58	47	40	47	106
257591 F	91	84	74	67	49	83	112

Triglyceride values were generally unaffected.

J. A. Klepfer
9/24/93

0.11 mg/kg

Add 8 hr time point.

09/24/1993

Test Article: 309132
Study No.: D06893
Start Date: 09/22/1993
Termination Date: 10/22/1993

TO: Mr. R. A. Byrd (Study Director/Project Leader)

PROTOCOL AMENDMENT

Approved by:

Study Director	R.A.	Byrd	09/24/1993 02:03:12PM
Sci. Supvsr.	N.R.	Bernhard	09/24/1993 02:07:36PM

AMENDMENT # 1
An appropriate blood glucose response was shown with the initial dose administration. A second dose administration will be given to further define the hypoglycemic response. The protocol is amended as follows:

Dose administration will occur September 27, 1993.

6. Dose Preparation

Prepare approximately 6 to 8 ml of solution at 1.1 mg/ml.

8. Animals

Each animal will receive 0.11 mg LY309132/kg. The dose volume will remain unchanged at 0.1 ml/kg.

15. Clinical Chemistry

Collect an additional blood sample from each dog at 8 hours following dose administration to determine blood glucose and triglycerides levels.

16. Biochemical Toxicology

Collect an additional blood sample from each dog at 8 hours following dose administration to determine serum concentration of LY309132.

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 5 (0-8 hour bleed)

Listed below are the glucose and triglyceride values (mg/dl) for samples collected over a 8 hour period.

Animal #		0hr	0.5hr	1.0hr	2.0hr	4.0hr	6.0hr	8.0hr
256051	GLU	75	71	87	92	90	84	83
	TRIG	19	37	47	45	38	60	53
256961	GLU	73	66	80	71	57	62	61
	TRIG	27	40	35	28	68	66	45
293071	GLU	71	78	72	80	66	65	53
	TRIG	24	44	44	37	48	48	35
255491	GLU	83	70	63	64	75	64	57
	TRIG	19	46	52	40	64	51	57
255691	GLU	73	65	60	61	60	62	66
	TRIG	33	47	43	50	68	69	68
257591	GLU	82	78	69	72	67	67	61
	TRIG	24	32	24	32	44	44	57

J. A. Klepfer
9/28/93

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 6 (24 hour bleed)

Listed below are the glucose and triglyceride values (mg/dl) for samples collected at the 24 hour collection inteval.

Animal #	GLU	TRIG
256051	71	32
256961	87	35
273071	105	26
255491	92	33
255691	97	41
257591	106	34

J. A. Klepfer
9/28/93

09/30/1993

Test Article: 309132
Study No.: D06893
Start Date: 09/22/1993
Termination Date: 10/22/1993

TO: Mr. R. A. Byrd (Study Director/Project Leader)

PROTOCOL AMENDMENT

Approved by:

Study Director	R.A.	Byrd	09/30/1993 07:39:17AM
Sci. Supvsr. (Alt)	C.M.	Murphy-Farmer	09/30/1993 07:49:05AM

AMENDMENT # 2
A third dose administration will be given to further explore the hypoglycemic response.

Dose administration will occur October 4, 1993.

6. Dose Preparation

Prepare approximately 6 to 8 ml of solution at 2 mg/ml.

8. Animals

Each animal will receive 0.2 mg LY309132/kg. The dose volume will remain at 0.1 ml/kg.

15 and 16. Clinical Chemistry and Biochemical Toxicology

Collect blood samples outlined in the original protocol and Amendment # 1 from each dog and an additional sample from each dog at 10 hours postdose to measure the parameters outlined in the respective sections.

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 11

Listed below are the glucose and triglyceride values (mg/dl) for samples collected Monday 10/4/93.

Animal #		0hr	0.5hr	1.0hr	2.0hr	4.0hr	6.0hr	8.0hr	10hr
256051	GLU	88	86	77	67	74	51	41	40
	TRIG	41	40	31	48	74	57	74	52
256961	GLU	78	78	66	67	41	58	52	72
	TRIG	43	37	15	35	68	67	69	62
273071	GLU	74	74	84	75	68	59	47	50
		43	39	30	45	52	58	50	50
255491	GLU	77	70	57	61	51	64	46	49
	TRIG	51	65	64	75	107	94	100	61
255691	GLU	79	72	68	56	36	43	40	45
	TRIG	37	52	36	61	69	75	64	67
257591	GLU	103	102	92	103	98	117	98	100
	TRIG	35	40	41	50	55	70	55	42

J. A. Klepfer
10/04/93

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 12

Listed below are the glucose and triglyceride values (mg/dl) for samples collected Tuesday 10/5/93.

Animal #		10hr	24hr
256051	GLU	40	86
	TRIG	52	25
256961	GLU	72	~~61~~ 90
	TRIG	62	~~17~~ 30
273071	GLU	50	61
		50	17
255491	GLU	49	84
	TRIG	61	36
255691	GLU	45	66
	TRIG	67	39
257591	GLU	100	93
	TRIG	42	29

J. A. Klepfer
10/06/93

10/08/1993

Test Article: 309132
Study No.: D06893
Start Date: 09/22/1993
Termination Date: 10/22/1993

TO: Mr. R. A. Byrd (Study Director/Project Leader)

PROTOCOL AMENDMENT

Approved by:

Study Director	R.A.	Byrd	10/08/1993 10:12:09AM
Sci. Supvsr.	N.R.	Bernhard	10/08/1993 10:20:39AM

AMENDMENT # 3
An appropriate blood glucose response was seen at 0.2 mg/kg. Phase IV will be conducted at 0.2 mg LY309132/kg with zinc contained in the lot of material.

The following sections are amended:

Dose Administration is scheduled for October 11, 1993 and live-phase termination is October 12, 1993.

5. Test Article

Lot No.: 2685-47A
Potency: 5.09 mg LY309132/vial
Zinc content: 0.0235 mg of zinc oxide/vial

6. Dose Preparation

Prepare dose solution (minimum of 6 ml) at 2 mg LY309132/ml with Humulin R diluent (Lot 6PG22A)

7 and 8. Dose Administration and Animals

Administer 0.1 ml/kg subcutaneously to each dog. Each animal will receive 0.2 mg LY309132/kg of body weight.

15 and 16. Clinical Chemistry and Biochemical Toxicology

Collect blood samples at 0, 0.5, 1, 2, 4, 6, 8, 10, and 24 following dose administration (same times as in Phase II).

cc:
Subj: Vials for Dose Ranging Studies

Richard,

We have assayed the vials to be used in your dose ranging studies. The vials
were made from the CT/Tox bulk lot 487EM3 by Mark Brader. One vial (lot
2685-47A) was formulated with Zn and the other vial (lot 2685-47B) was formulated with no Zn. These assay results are as follows:

Lot 2685-47A	5.09 mg LY309132/vial	2.4% total rel. sub. by RP chrom.
Lot 2685-47B	5.15 mg LY309132/vial	2.5% total rel. sub. by RP chrom.

If you have any questions please call.

Dean Clodfelter
6-6130

Press RETURN for more...

LMail>
#370 5-OCT-1993 13:49:24.15 MAIL

From: CLODFELTER DEAN K (MCVAX0::CLODF)

To: BYRD RICHARD A (GLVAX0::BYRD)
cc: BECKAGE MICHAEL J (MCVAX0::RC30960)
BRADER MARK L (MCVAX0::RC86290)
BAKER JEFFREY C (MCVAX0::EB65660)
MILES CYNTHIA D (INDYVM1::RZ81205)

LMail>

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 15

Listed below are the glucose and triglyceride values (mg/dl) for samples collected Monday 10/11/93.

Animal #		0hr	0.5hr	1.0hr	2.0hr	4.0hr	6.0hr	8.0hr
256051	GLU	74	77	79	63	74	58	43
	TRIG	34	52	54	56	65	70	60
256961	GLU	84	77	66	63	48	63	71
	TRIG	48	72	82	37	93	53	55
273071	GLU	86	85	81	84	73	69	47
		32	52	38	42	53	47	48
255491	GLU	91	68	75	67	75	63	39
	TRIG	44	55	60	68	77	80	69
255691	GLU	72	63	67	37	36	50	41
	TRIG	45	58	61	71	78	86	70
257591	GLU	82	84	91	72	66	82	61
	TRIG	45	54	41	29	73	61	48

J. A. Klepfer
10/12/93

Subj: C16 Material for GLP Tox Studies

From: NAME: BYRD RICHARD A
FUNC: GL991
TEL: 277-4853 <BYRD RICHARD A@A1@GLVAX0>
To: NAME: BRADER MARK L <RC86290@MRGATE@MCVAX0>
CC: NAME: BECKAGE MICHAEL J <RC30960@MRGATE@MCVAX0>,
NAME: BAKER JEFFREY C <EB65660@MRGATE@MCVAX0>,
NAME: STCLAIR ROGER L <STCLAIR ROGER L@MSGR@CRPVAX>,
NAME: ZIMMERMANN JOHN L <ZIMMERMANN JOHN L@MSGR@CRPVAX>

We received our Zn formulated C16 yesterday around 4:00 pm. We noticed that the material was shipped at ambient temperature. We have been storing C16 at -20 degrees. Is that necessary??

Press RETURN for more...

LMail>
#379 6-OCT-1993 09:58:32.57 MAIL
Roger St Clair pointed out to me that the individual vials were not labeled with respect to compound number and lot number; this will not be a problem for our dose range studies. However, he told me that the material for the GLP studies (i.e., the material we are getting in November) must have labels on each vial; otherwise, Quality Assurance will write us up as being out of compliance. Perhaps, you (or someone else if its more appropriate) should confirm the necessary info with Roger.

Thanks.

RAB

INTERIM STUDY SUMMARY REPORT
Clinical Pathology

Compound: 309132
Study #: D06893
Project Leader: R. A. Byrd
Project Pathologist: none assigned
Clinical Pathologist: J. P. McGrath
Day: 16

Listed below are the glucose and triglyceride values (mg/dl) for samples collected Monday 10/12/93.

Animal #		10hr	24hr
256051	GLU	43	94
	TRIG	46	37
256961	GLU	47	97
	TRIG	62	47
273071	GLU	33	65
		44	38
255491	GLU	41	72
	TRIG	56	36
255691	GLU	57	78
	TRIG	70	53
257591	GLU	55	109
	TRIG	54	36

J. A. Klepfer
10/13/93

Clinical Signs

A technician observed each dog frequently during the day of each dose administration and again the following morning at approximately 24 hours postdose.

Body Weights

Each dog was weighed prior to each dose administration.

Food Consumption

Approximately 150 g of Purina Certified Canine Diet 5007 was offered to each dog approximately 30 minutes prior to dose administration and another 150 g immediately following the dose. The amount of food that was not consumed after 24 hours was estimated for each animal.

Clinical Chemistry

Blood samples were collected to determine glucose and triglycerides concentrations at 0, 0.5, 1, 2, 4, 6, and 24 hours following each dose administration. Additional blood samples were collected from each dog at 8 hours postdose during Phase II, and at 8 and 10 hours postdose during Phases III and IV.

Blood Collection to Measure Serum Concentrations of LY309132

Approximately three ml of blood were collected from the jugular vein of each dog at 0, 0.5, 1, 2, 4, 6, and 24 hours following each dose administration. Additional blood samples were collected from each dog at 8 hours postdose during Phase II, and at 8 and 10 hours postdose during Phases III and IV.

Following blood collection, each sample was delivered to Sample Processing for serum removal. The serum samples were stored frozen at -70 degrees C until sent to the analyst.

RESULTS

Survival

All dogs survived the live-phase portion of the study.

Clinical Observations and Food Consumption

No clinical signs were seen and no change in appetites observed during the live-phase portion of the study.

Plasma Concentration of Compound 282072

Results will be provided by the analyst.

N. R. Bernhard
Scientist Supervisor

Compound: LY309132

Study Number: D06893

Protocol Title: A Dose Ranging Study with LY309132 Administered Subcutaneously to Beagle Dogs

Report Type: Clinical Pathology

Methods

Blood samples for glucose and triglyceride determinations were obtained from each animal at 0, 0.5, 1, 2, 4, 6, and 24 hours after dose administration on Day 0, at 0, 0.5, 1, 2, 4, 6, 8, and 24 hours after dose administration on Day 5, and at 0, 0.5, 1, 2, 4, 6, 8, 10, and 24 hours after dose administration of Days 12 and 19.

Clinical Chemistry

Blood samples were collected into a tube containing no anticoagulant. Serum was obtained by centrifugation and values for the following parameters were determined.

Parameter (Abbreviation)	Unit	Method
Glucose (GLU)	Milligrams/deciliter (MG/DL)	Hexokinase[1]
Triglyceride (TRIG)	Milligrams/deciliter (MG/DL)	Lipase[1]

[1]Monarch ™Chemistry System. Instrumentation Laboratory, Inc., Lexington, MA.

Statistical Analysis

Quantitative results were analyzed for each sex at each time point with a one-factor analysis of variance. Linear treatment contrasts were tested in a sequential fashion (Tukey *et al.*, 1985) to evaluate dose response.

Results

Clinical Chemistry

Administration of LY309132 at 0.07 mg/kg resulted in a slight decrease in mean blood glucose (*ca* 9%) at 30 minutes post-injection. Thereafter, mean blood glucose fluctuated and continued to decrease, reaching its maximum drop (*ca* 31 and 43% in males and females, respectively) by 4 hours post-injection, compared to the pretreatment values. Mean blood glucose began to rise thereafter, and reached above the pretreatment values (*ca* 26%) at 24 hours post-injection.

Increasing the dose to 0.11 mg/kg resulted in less remarkable changes in mean blood glucose levels, compared to the earlier dose of 0.07 mg/kg. Maximum decrease in mean blood glucose (*ca* 17%) occurred at 8 hours post-injection. By 24 hours post-injection, mean blood glucose level was above the pretreatment values (*ca* 27%).

Administration of LY309132 at 0.2 mg/kg resulted in progressive decrease in mean blood glucose levels. An initial minimal decrease (*ca* 4%) at 30 minutes post-injection reached its maximum drop in males (*ca* 44%) and females (*ca* 33%) by 8 hours and 4 hours post-injection, respectively, compared to the pretreatment values. Thereafter, mean blood glucose level began to rise, but remained below the pretreatment values (*ca* 4%) by 24 hours post-injection in both males and females.

October 27, 1993

Archives
cc: Mr. R. A. Byrd

Summary of Study D06893 - LY309132 (B29(C16) human insulin analog)

INTRODUCTION

Study D06893 was conducted to explore a range of doses with LY309132 to determine toxicity, blood glucose and triglyceride levels, and to measure blood plasma concentrations following subcutaneous dose administrations. These data will be used to select doses for a future dog study.

MATERIAL AND METHODS

Test Animals, Treatment Group, and Live-Phase Duration

The study had one treatment group containing three male and three female beagle dogs. The same group of dogs were used during each phase of the study. The dogs were selected from the acute stock colony. Each animal had been assigned to previous studies but had not received any experimental compounds for at least 2 months prior to this study. There were four dosing phases. The duration of each phase was 24 hours following each dose administration. Dosing occurred September 22, September 27, October 4, and October 11, 1993 for Phases I, II, III, and IV, respectively. The live-phase portion of the study terminated October 12, 1993.

Dose Preparation

Lot RS0163 of LY309132 was used during Phases I, II, and III and had a potency of 4.91 mg LY309132/vial. Lot 2685-47A containing 5.09 mg LY309132/vial with 0.0235 mg of zinc oxide in each vial was used for Phase IV. Dose solutions were prepared at 0.7, 1.1, 2, and 2 mg LY309132/ml with Humulin R diluent for Phases I, II, III, and IV, respectively. Humulin R diluent, lot 6PG22A, was used in preparing dose solutions throughout the study.

Dose Administration

A single dose was given subcutaneously to each dog during each phase of the study. Doses administered to each dog were 0.07, 0.11, 0.2, and 0.2 mg LY309132/kg of body weight during Phases I, II, III, and IV, respectively. The dose volume remained constant at 0.1 ml/kg of body weight. The dose volume for each dog was calculated from a body weight taken prior to each phase. The dose was given into the dorsal neck area of each dog.

Addition of zinc to LY309132 (0.2 mg/kg) revealed relatively similar changes, as described earlier for the 0.2 mg/kg dose without zinc. By 24 hours post-injection, however, mean blood glucose level for the males was below (*ca* 8%), but for the females was above (*ca* 19%) the pretreatment values.

In summary, maximum effects on blood glucose level occurred following the administration of 0.2 mg/kg LY309132 with or without addition of zinc.

Administration of LY309132 resulted in an overall dose-related increase in mean serum triglycerides. The increases were largely noted at 30 minutes post-injection and were either progressive, fluctuated or sustained through the next 6 hours post-injection. The increases in mean serum triglycerides became comparatively less remarkable thereafter, and the mean values were either comparable to or less than the pretreatment values by 24 hours post-injection. The increases in mean serum triglycerides following the administration of 0.11 mg/kg LY309132 were exaggerated, because of the pretreatment values which were lower than the established reference ranges for the species.

References

TUKEY, J. W., CIMINERA, J. L., and HEYSE, J. F. (1985). Testing the statistical certainty of response to increasing doses of a drug. Biometrics 41, 295-301.

A. Deldar, D.V.M., Ph.D.
Senior Clinical Pathologist

April 7, 1994
Date

Calendar 1993

January

S	M	T	W	T	F	S
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30
31						

February

S	M	T	W	T	F	S
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28						

March

S	M	T	W	T	F	S
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28	29	30	31			

April

S	M	T	W	T	F	S
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	

May

S	M	T	W	T	F	S
						1
2	3	4	5	6	7	8
9	10	11	12	13	14	15
16	17	18	19	20	21	22
23	24	25	26	27	28	29
30	31					

June

S	M	T	W	T	F	S
		1	2	3	4	5
6	7	8	9	10	11	12
13	14	15	16	17	18	19
20	21	22	23	24	25	26
27	28	29	30			

July

S	M	T	W	T	F	S
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

August

S	M	T	W	T	F	S
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

Sept mber

S	M	T	W	T	F	S
			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29	30		

October

S	M	T	W	T	F	S
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30
31						

November

S	M	T	W	T	F	S
	1	2	3	4	5	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28	29	30				

December

S	M	T	W	T	F	S
			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29	30	31	